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## CD4<sup>+</sup> T-Cell Responses to Epstein-Barr Virus Nuclear Antigen EBNA1 in Chinese Populations Are Highly Focused on Novel C-Terminal Domain-Derived Epitopes

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**Epstein-Barr virus nuclear antigen EBNA1, the one viral protein uniformly expressed in nasopharyngeal carcinoma (NPC), represents a prime target for T-cell-based immunotherapy. However, little is known about the EBNA1 epitopes, particularly CD4 epitopes, presented by HLA alleles in Chinese people, the group at highest risk for NPC. We analyzed the CD4<sup>+</sup> T-cell responses to EBNA1 in 78 healthy Chinese donors and found marked focusing on a small number of epitopes in the EBNA1 C-terminal region, including a DP5-restricted epitope that was recognized by almost half of the donors tested and elicited responses able to recognize EBNA1-expressing, DP5-positive target cells.**

Undifferentiated nasopharyngeal carcinoma (NPC), an unusually prevalent tumor in Southeast Asian and particularly Chinese populations, is consistently Epstein-Barr virus (EBV) positive and expresses a subset of EBV latent proteins. These include the nuclear antigen EBNA1, the latent membrane protein LMP2 in most cases, and in some cases LMP1 (12). A detailed knowledge of both CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses to these antigens is needed if one is to exploit such responses for immunotherapeutic use (1, 7, 15). While there has been extensive work on CD8<sup>+</sup> T-cell responses in that regard (4), CD4-based studies have focused almost exclusively on Caucasian donors (3, 6, 8, 10, 11, 14, 20) and little is known about responses restricted through the different array of HLA II alleles found in the Chinese population. Here we focus on CD4 responses to EBNA1, the only viral protein known to be expressed in all NPC tumors and one which proves to be a rich source of CD4 epitopes.

**ELISPOT assay mapping of CD4 epitope regions.** Eighty-one peptides (20-mers overlapping by 15 residues) were synthesized according to the EBNA1 sequence common to 27/31 Chinese EBV strains, of which the NPC 15 (CKL) virus strain is the prototype (9). On the basis of published protocols (6), these peptides were tested individually in enzyme-linked immunospot (ELISPOT) assays of gamma interferon (IFN- $\gamma$ ) release with peripheral blood mononuclear cells (PBMCs, CD8<sup>+</sup> T cell depleted) obtained with informed consent from healthy, EBV-seropositive Chinese donors resident in Hong Kong, an area with a high incidence of NPC. Figure 1 shows representative results from four responsive donors, HK 201,

HK 215, HK 263, and HK 280, to illustrate the low backgrounds (0 to 10 spots/well) usually seen and the clear focusing of responses mostly on adjacent sets of two or three overlapping peptides in specific regions of the molecule. Overall, 50/78 donors tested in repeat assays showed reproducible responses to particular peptides.

On this basis, we identified 10 distinct epitope regions in the Chinese EBNA1 sequence. These are located on a linear map of the EBNA1 protein (Fig. 2, right) where the filled horizontal bars represent the percentages of donors responding to each epitope region. Alongside (Fig. 2, left) are shown the corresponding data from 37 healthy seropositive Caucasian donors tested against the standard B95.8 EBNA1 peptide panel (6; unpublished data). In both cases, CD4 epitopes are concentrated within the C-terminal half of the molecule. However, compared to those of Caucasians, the EBNA1 responses of Chinese donors are focused on a smaller number of epitope regions, with correspondingly higher percentages of donors responding to individual regions. In particular, EBNA1 peptides 67 and 68 were recognized by 47% (37/78) of the Chinese donors tested, a far higher frequency than that seen for any Caucasian donor response.

**Epitope definition by CD4<sup>+</sup> T-cell cloning.** Four epitope regions, represented by Chinese EBNA1 peptides 48 and 49, 50 and 51, 56 and 57, and 67 and 68, were selected for detailed analysis. On the basis of previously published protocols (8), PBMCs from selected donors were stimulated *in vitro* with relevant peptide pairs and cloned on day 7 at limiting dilution (0.3 cell/well) on autologous CKL virus-transformed B-lymphoblastoid cell line (LCL) feeders, and clones reactive to the stimulating peptide pair were identified by specific IFN- $\gamma$  release in an enzyme-linked immunosorbent assay. Such clones were assayed on the autologous LCL loaded with serial dilutions of individual peptides from the epitope region in order to identify the optimal 20-mer epitope. Representative results are

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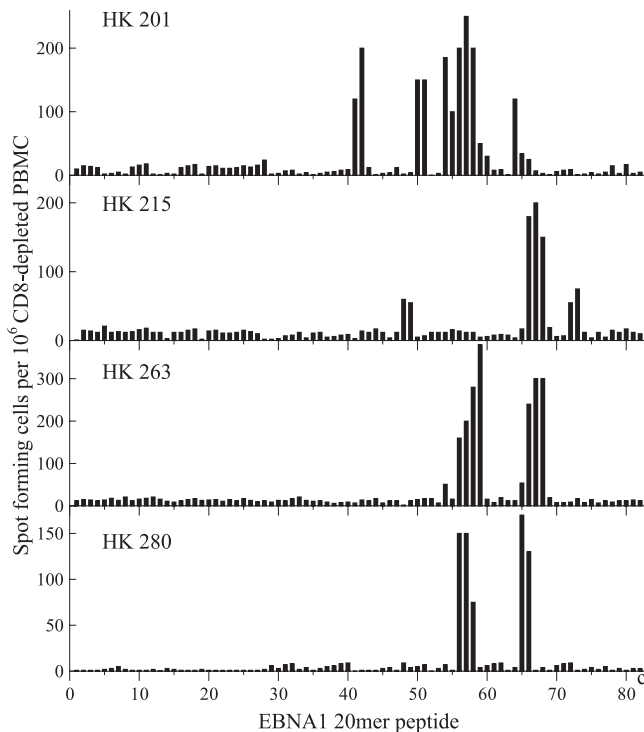


FIG. 1. Results of IFN- $\gamma$  ELISPOT assays using CD8-depleted PBMCs from healthy Chinese donors exposed to individual CKL strain EBNA1 peptides (1 to 81) or assayed in the absence of peptide as a control (c). Results from four different donors are shown as numbers of spot-forming cells per  $10^6$  CD8-depleted PBMCs tested. Note that responses occasionally mapped to a single peptide (e.g., HK 201, peptide 64) but usually mapped to two or three adjacent peptides (e.g., HK 201, peptides 41 and 42 and peptides 50 and 51; HK 215, peptides 48 and 49 and peptides 66, 67, and 68). Where there was recognition of four or five adjacent peptides, cloning frequently revealed coresident responses to two separate epitopes.

shown in Fig. 3A. Clones from donor HK 215 against the peptide 48 and 49 region recognized peptide 49 but, surprisingly, not peptide 48 even though both peptides had elicited equally strong responses in the original ELISPOT assays. Clones against the other epitope regions showed recognition of two or more adjacent peptides but consistently identified one of those peptides most efficiently, i.e., peptide 50 for clones from donor HK 228, peptide 56 for clones from donor HK 233, and peptide 67 for clones from two donors, HK 215 and HK 228 (data not shown). Note also that these EBNA1 epitope-specific clones all showed functional avidities (defined as the minimum peptide concentration required for 50% monoclonal IFN- $\gamma$  release) within the 10- to 100-nM range, similar to those seen for most EBNA1-specific reactivities isolated from Caucasian donors (8). Moreover, they were also confirmed as antigen specific since they recognized autologous target cells expressing a class II-targeted EBNA1 protein expressed from a recombinant vaccinia virus vector (17; data not shown).

**HLA class II restriction of epitope-specific responses.** Figure 3B shows the restriction data obtained from these clones in two types of assay. As previously described (8), these used (i) individual HLA-DP-, -DQ-, or -DR-specific monoclonal anti-

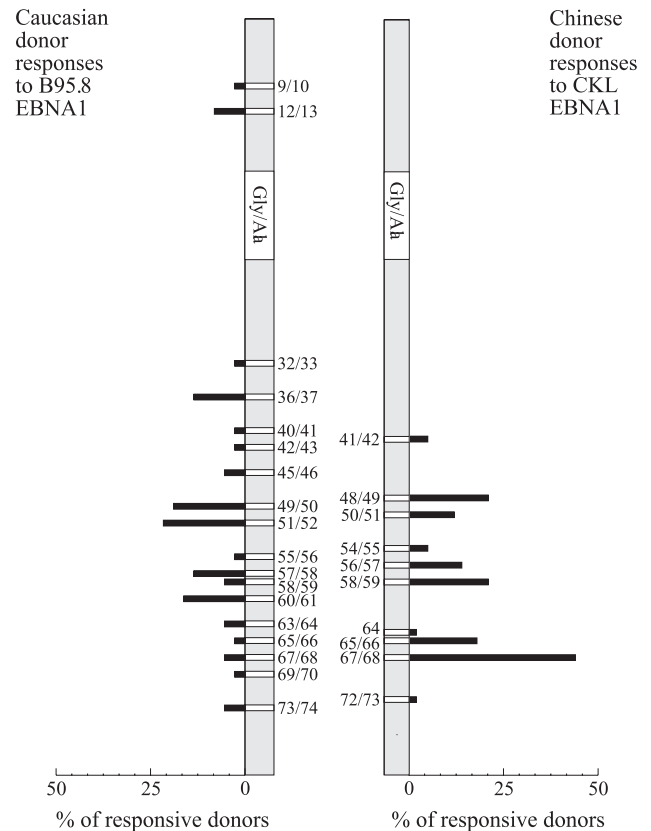


FIG. 2. Locations of CD4 epitope regions, identified by peptide number, on a linear map of the EBNA1 protein (with the position of the internal glycine-alanine repeat domain also shown). (Right) Summary of data from 78 healthy Chinese donors screened by using the CKL strain EBNA1 peptide panel. (Left) Summary of data from 37 healthy Caucasian donors from the United Kingdom who were screened by using the B95.8 strain EBNA1 peptide panel. In each case, the filled bars indicate the percentages of donors tested who responded to these particular epitope regions.

bodies (MAbs) to block the recognition of autologous peptide-loaded cells and (ii) allogeneic target cells of known HLA type as peptide-presenting cells. Both the peptide 49-specific and peptide 56-specific clones proved to be HLA-DR restricted by blocking, but the precise alleles could not be identified by using the allogeneic targets available at that time. We therefore HLA typed 47 donors from the original panel; this revealed strong correlations between HLA-DR51 positivity and responsiveness to peptide 49 and between HLA-DR11 positivity and responsiveness to peptide 56, suggesting that these were the likely restricting alleles. With regard to peptide 50, the MAb blocking and allogeneic LCL assays suggested that this was HLA-DQ7 restricted and, indeed, all five donors showing this response in ELISPOT assays were DQ7 positive; however, another 15 DQ7-positive individuals showed no response, suggesting that this is not a consistently immunodominant epitope. Most importantly, the peptide 67 response proved to be HLA-DP5 restricted. This immediately suggested why it was seen so frequently in the ELISPOT assays, since HLA-DP5 is carried by >50% of Chinese people (19) and, indeed, all responders to this allele who were HLA typed proved to be DP5 positive.

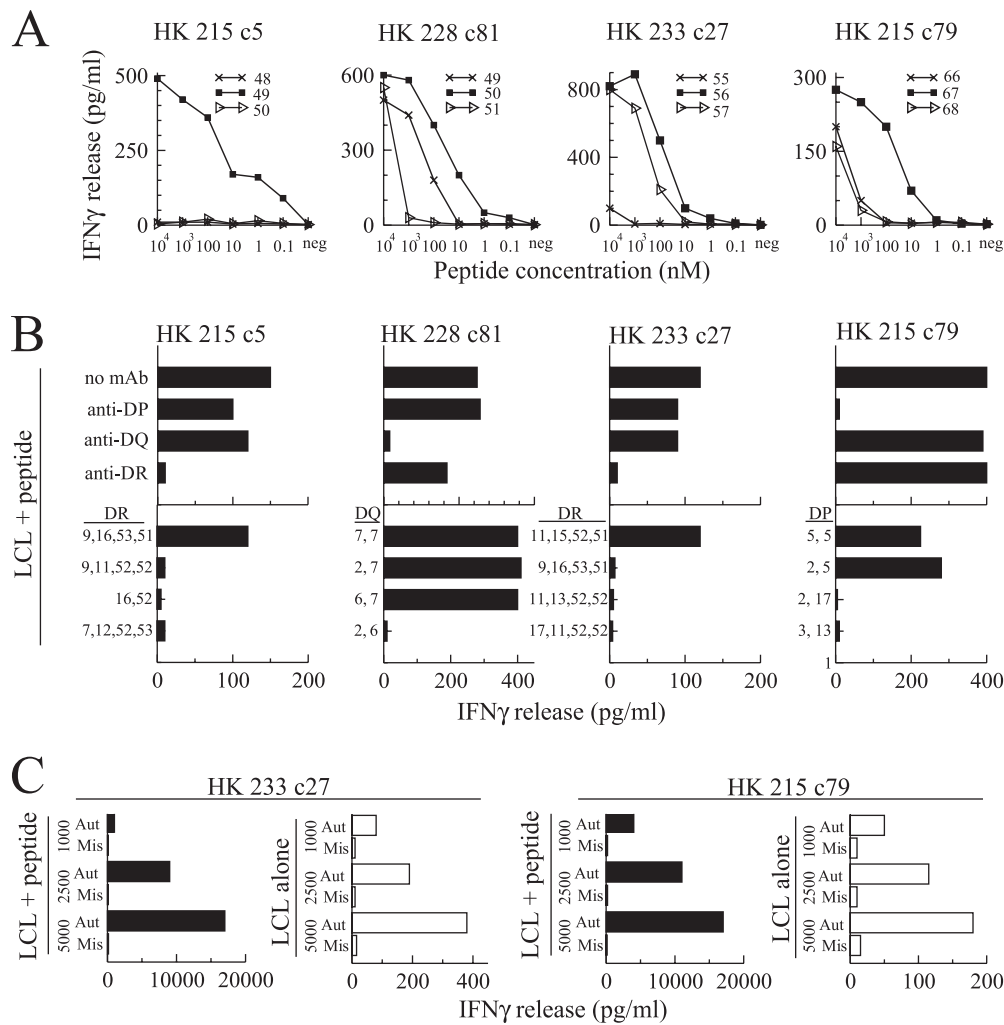


FIG. 3. Functional analysis of CD4<sup>+</sup> T-cell clones established from selected healthy Chinese donors by stimulating PBMCs with CKL strain EBNA1 peptide pair 48 and 49, 49 and 50, 56 and 57, or 67 and 68. (A) Identifying the optimal 20-mer epitope. Clones were stimulated with the autologous LCL preloaded with limiting concentrations of individual peptides from the epitope region; recognition was assayed as IFN-γ release by enzyme-linked immunosorbent assay. neg, negative. (B) Identifying HLA class II restriction. (Top) Clones were stimulated with the autologous LCL preloaded with a just-optimal concentration of the epitope peptide in the presence of MAb to HLA-DP, -DQ, or -DR. (Bottom) Clones were stimulated with the autologous LCL or with an HLA-typed allogeneic LCL preloaded with a concentration of peptide similar to that above; relevant HLA class II alleles shared between the LCL and donor T cells are shown. Results are expressed as in panel A. (C) Recognizing unmanipulated LCL. Clones seeded at different cell numbers were stimulated with a standard number of cells of an autologous LCL (Aut) or an allogeneic HLA-mismatched LCL (Mis), either alone or after loading with an optimal concentration of epitope peptide, and recognition was assayed as above.

TABLE 1. Summary of EBNA1 epitope responses

EBNA1 peptide no.	Amino acid coordinates	Epitope sequence <sup>a</sup>	No. of responders <sup>b</sup>	Restriction element	% LCL recognition <sup>c</sup>
41/42	434–458	IEQGPTDDPGEGPSTGPRGQGDGGR	2/43	ND <sup>e</sup>	ND
49	474–493	SNPKFENIAEGLRVLLARSH	16/78	DR51	<0.2
50	479–498	ENIAEGLRVLLARSHVERTT	5/43	DQ7	<0.2
54/55	499–523	EEGNWVAGVFVYGGSKTSLYNLRRG	4/78	ND	ND
56	509–528	VYGGSKTSLYNLRRGIALAV	11/78	DR11 <sup>d</sup>	2
58/59	519–543	NLRRGIALAVPQCRITPLSRLPFGM	16/78	ND	ND
64	549–568	POPGPLRESIVCYFMVFLQT	1/43	ND	ND
65/66	554–578	LRESIVCYFMVFLQTHIFAEVLKDA	14/78	ND	ND
67	564–583	VFLQTHIFAEVLKDAIKDL	34/78	DP5	1
72/73	589–613	PTCNIKVTVCSFDDGVDLPPWFPPM	1/43	ND	ND

<sup>a</sup> Amino acid changes in CKL strain EBNA1 relative to B95.8 EBNA1 are underlined.  
<sup>b</sup> Note that of the 78 donors used, 43 were tested on all 81 EBNA1 peptides and a further 35 were tested on a limited panel of peptides that did not include peptide 41, 42, 50, 64, 72, or 73.  
<sup>c</sup> ND, not determined.  
<sup>d</sup> DR-restricted responses where the restricting allele is inferred to be DR51 and DR11 from the HLA type of epitope-responsive donors.  
<sup>e</sup> IFN-γ levels from CD4<sup>+</sup> T cells challenged with the autologous LCL are expressed as a percentage of the levels seen on challenge with the same LCL optimally loaded with epitope peptide.

**CD4<sup>+</sup> T-cell recognition of latently infected cells.** Interest in CD4<sup>+</sup> T-cell responses to EBV latent proteins reflects not just their likely importance in the maintenance of effective CD8<sup>+</sup> T-cell surveillance (2, 13, 16) but also their possible role as direct effectors capable of recognizing latently infected cells that, like NPC tumors (5, 18), express HLA class II molecules. To investigate this latter point, we used published protocols (8) to assay the EBNA1-specific clones for recognition of autologous LCL targets. Specific recognition could not be detected with clones to peptide 49 or 50 (data not shown). However, clones to peptides 56 and 67 clearly did recognize the autologous LCL, with recognition titrating against T-cell input; for these clones, levels of IFN- $\gamma$  produced in response to the autologous LCL (Fig. 3C, open bars, LCL alone) were around 2% and 1%, respectively, of those produced in response to the same target cells optimally loaded with epitope peptide (Fig. 3C, filled bars, LCL + peptide).

Table 1 summarizes the essential data from the present study. We conclude that most healthy Chinese donors possess a strong CD4<sup>+</sup> T-cell memory for EBNA1; that responses are focused on a small number of C-terminal EBNA1 epitopes, including a DP5-restricted epitope seen by almost 50% of the donors tested; and that some of the above responses (including that to the DP5 epitope) have the potential to directly recognize a latently infected HLA II-positive LCL. These findings will help in both the design and testing of immunotherapeutic strategies targeting NPC, whether based on vaccines to stimulate the patient's own responses (7, 17) or on the adoptive transfer of in vitro-expanded T-cell populations (1, 15).

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